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Methods in Enzymology

Volume 65

Nucleic Acids

Part I

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1980

ACADEMIC PRESS, INC.
(Harcourt Brace Jovanovich, Publishers)
Orlando San Diego New York London
Toronto Montreal Sydney Tokyo

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
Main entry under title:

Nucleic acids.

(Methods in enzymology, v. 12, 20–21, 29–30, 65)
Pts. C, E–F have title: Nucleic acids and protein synthesis: with editor's names in reverse order on t. p.
Includes bibliographical references.
1. Nucleic acids. 2. Protein biosynthesis.
I. Grossman, Lawrence, Date ed. II. Moldave, Kivie, Date ed. III. Title: Nucleic acids and protein synthesis. IV. Series: Methods in enzymology. v. 12 [etc.] [DNLM: 1. Nucleic acids––Biosynthesis. 2. Proteins––Biosynthesis. W 1 Me9615K v. 30 1974 QU 55 N964 1974]
QP601.M49 vol. 12, etc. 574.1'925'08s [QP620]
ISBN 0–12–181965–5 (v. 65) [574.8'732] 74–26909

PRINTED IN THE UNITED STATES OF AMERICA

85 86 87 88 9 8 7 6 5 4 3

CONTRIBUTORS TO

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phase variation in *Salmonella*,[27] the herpes simplex genome[28] and the 2 micron circle in *Saccharomyces cerevisiae*.[29] A diagnostic feature of flip-flop is the presence of less than stoichiometric amounts of fragments produced by a restriction site located asymmetrically in the invertible segment and a site located outside of the invertible segment. This principle is illustrated by the fragments generated from bacteriophage Mu DNA by the enzymes which cut within and outside of the invertible G segment.[9,30,31] Figure 7 shows the *Kpn*I-*Pst*I digests of Mu DNAs. *Kpn*I site is located asymmetrically within G whereas the *Pst*I site is outside of G. Two different fragments can be seen when the DNA molecules have the G segment in both orientations. When G is only in one orientation, only one fragment specific to a particular orientation can be seen.

[27] J. Zieg, M. Silverman, M. Hilmen, and M. Simon, *in* "The Operon" (J. H. Miller and W. S. Reznikoff, eds.), p. 411. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1978.
[28] B. Roizman, *Cell* 16, 481 (1979).
[29] J. R. Broach, J. F. Atkins, C. McGill and L. Chow, *Cell* 16, 827 (1979).
[30] D. Kamp, R. Kahmann, D. Zipser, T. R. Broach, and L. T. Chow, *Nature* (*London*) 271, 577 (1978).
[31] A. Toussaint, N. Lefebvre, J. R. Scott, J. A. Cowan, F. DeBruijn, and A. I. Bukhari, *Virology* 89, 146 (1978).
[32] M. Magazin, M. Howe, and B. Allet, *Virology* 77, 677 (1977).

[53] Determination of Fragment Order through Partial Digests and Multiple Enzyme Digests

By KATHLEEN J. DANNA

Many applications of restriction endonuclease cleavage of DNA are possible only if the resulting fragments have been ordered to produce a physical map. This article describes the basic principles of two techniques for fragment ordering: analysis of partial digestion products and multiple enzyme digestion. These were the first methods used to determine a physical map of a DNA genome, the simian virus 40 (SV40) genome,[1] and remain perhaps the most straightforward and easy to interpret procedures. Moreover, aside from apparatus for DNA fragment analysis, these approaches require only the DNA and restriction endonuclease(s) of interest.

Ordering DNA fragments by partial endonuclease digestion is analogous to a sequencing technique for RNA described by Adams *et al.*,[2] who

[1] K. J. Danna, G. H. Sack, Jr., and D. Nathans, *J. Mol. Biol.* 78, 363 (1973).
[2] J. M. Adams, P. G. N. Jeppesen, F. Sanger, and B. G. Barrell, *Nature* (*London*) 223, 1009 (1969).

ISBN 0-12-181965-5

used partial T1 ribonuclease digestion products of R17 RNA to order T1 oligonucleotides of the limit digest. A partial digest of DNA is obtained by limiting the reaction time so that the endonuclease does not cleave at all possible recognition sites in the DNA. Thus, partial digestion yields some fragments comprised of two or more contiguous complete digestion products. By purifying a partial digestion product, incubating it with excess enzyme to complete the digestion, and identifying the resultant fragments, one can determine which final products are contained in a given partial digestion product. Analysis of several partial digestion products in this way enables one to deduce the order of all fragments of the limit digest.

Multiple enzyme digestion for ordering DNA fragments employs an approach routinely used for sequencing proteins and RNA, namely, sequential digestion with enzymes of different specificity. For DNA, the cleavage products of one endonuclease are characterized with respect to size and are then digested with a second endonuclease. Analysis of the resultant double-digestion products establishes the relationship between the cleavage sites of the two enzymes.

The partial digest and multiple enzyme digest approaches to fragment ordering are best illustrated by example. This chapter presents a model study which develops a physical map of the SV40 genome. Section I describes procedures for digestion of DNA with endonuclease and for analysis of cleavage products, with emphasis on techniques, such as polyacrylamide gel electrophoresis and autoradiography, that are used in the model study. Ordering fragments through analysis of partial digestion products is illustrated in Section II for the two sets of SV40 DNA fragments produced by cleavage with *Hin*cII and with *Hin*dIII. In Section III, *Taq*I and *Bam*H1 are used in multiple enzyme digestions with *Hin*cII and *Hin*dIII to generate a complex physical map that includes the cleavage sites for all four enzymes.

I. Basic Procedures

A. Digestion of DNA with Restriction Endonucleases

The first step in ordering DNA fragments is complete digestion of DNA with the endonuclease(s) of choice. Optimal reaction conditions (e.g., pH, salt concentrations, and temperature) for specific restriction endonucleases are described in the catalogues published by suppliers[3] and

[3] Bethesda Research Laboratories, Rockville, Maryland; Boehringer Mannheim, Indianapolis, Indiana; Miles Laboratories, Inc., Elkhart, Indiana; New England Biolabs, Beverly, Massachusetts.

elsewhere in this volume.[4] However, the amount of enzyme[5] needed to yield a limit digest must be determined empirically because the number of cleavage sites for a particular endonuclease in a given species of DNA cannot be predicted. A series of pilot reactions, in which both the ratio of enzyme to DNA and the incubation time are varied, is useful for determining the amount of enzyme needed to attain complete digestion. In the model study, each pilot reaction contained 0.2 μg of SV40 DNA in a 20-μl volume with either 0.1 unit, 0.5 unit, or 1 unit of enzyme. A 5-μl aliquot was withdrawn from each reaction mixture at 30 min, 1 hr, 2 hr, and 3 hr, and reaction in each was stopped by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% (w/v). Samples were then analyzed electrophoretically, as described in Section I,B, to determine which conditions resulted in complete digestion.

An important characteristic of a limit digest is that all cleavage products are equimolar. Therefore, when uniformly labeled [^{32}P]DNA is cleaved, the amount of radioactivity in each limit product is directly proportional to its size. Complete digestion can be verified by the addition of more endonuclease to a reaction mixture and incubation for a longer time. If digestion is complete, neither the amounts nor the sizes of the products will change.

The same methods can be used to establish conditions for partial digestion. Short reaction times result in large fragments that contain several contiguous complete digestion products, and longer times result in smaller fragments. A preparation of partial digestion products including fragments of all sizes can be obtained by combining several reaction mixtures incubated for different lengths of time.

Preparative reaction mixtures should be exactly scaled to pilot reactions that yield a high proportion of the desired products. A preparative digest containing 1×10^6 to 2×10^6 dpm of [^{32}P]DNA proved sufficient to map SV40 DNA, which is about 5000 nucleotide pairs in length.

B. Analysis of Cleavage Products

DNA fragments produced by restriction endonucleases have been separated by reverse phase chromatography,[6] hydroxylapatite chromatography,[7] agarose gel electrophoresis,[8,9] and polyacrylamide gel

[4] See this volume, Section III.
[5] For example, New England Biolabs defines 1 unit of enzyme as the amount that completely digests 1 μg of phage λ DNA in 15 min at the optimal temperature of incubation.
[6] R. D. Wells *et al.*, this volume, Article [41].
[7] D. Davis *et al.*, this volume, Article [49].
[8] P. A. Sharp, B. Sugden, and J. Sambrook, *Biochemistry* 12, 3055 (1973).
[9] R. C. Parker and B. Seed, this volume, Article [44].

electrophoresis.[10,11] For most of the analyses in the model study, vertical slab gels of polyacrylamide were used because of their high resolving power and high capacity. Visualization of DNA fragments in gels has been achieved by the use of both fluorescent[12] and nonfluorescent stains,[13] by the use of tungstate screens,[14] and by autoradiography.[15] In the model study, fragments of [^{32}P]DNA (specific activity of 5×10^5 dpm/μg of DNA) were visualized by autoradiography, a sensitive method that allows as little as 10^{-3} μg of DNA to be observed in 16 hr. Detailed descriptions of both slab gel electrophoresis and autoradiography have been presented in this series.[9–11,14,16] The remainder of this section reviews only the specific techniques used to prepare the slab gels, samples, and wet- and dried-gel autoradiograms for the model study.

Slab gels (14-cm wide, 13-cm long, 1-mm thick) are routinely prepared by the method of Loening[17] from these stock solutions:

1. Acrylamide (recrystallized from ethyl acetate), 15% (w/v)-*N,N'*-methylenebisacrylamide (recrystallized from acetone), 0.75% (w/v)
2. 10× electrophoresis buffer: 0.4 *M* Trizma base, 0.2 *M* sodium acetate, 0.02 *M* sodium EDTA, adjusted to pH 7.8 with glacial acetic acid
3. Ammonium persulfate, 5% (w/v), freshly made
4. *N,N,N',N'*-tetramethylethylenediamine (TEMED), neat

For a 4% polyacrylamide gel (total volume 40 ml), 10.7 ml of stock acrylamide solution are mixed with 4 ml of 10× electrophoresis buffer and 24.9 ml of deionized water. Polymerization is catalyzed by the addition of 0.42 ml of 5% ammonium persulfate and 0.042 ml of TEMED. The solution is poured between two glass plates, as described by DeWachter and Fiers,[16] to form the slab gel.

Prior to electrophoresis, DNA samples containing 1% SDS (w/v) are incubated at 37° for 10 min to disrupt protein–DNA aggregates. Samples are then made 10% (w/v) in sucrose and 0.02% (w/v) in bromphenol blue, are layered into wells in the gel, and are electrophoresed at constant voltage in a buffer of 0.04 *M* Trizma base, 0.02 *M* sodium acetate, 0.002 *M* sodium EDTA, adjusted to pH 7.8 with glacial acetic acid.

The time of electrophoresis and voltage required depend on the range of fragment sizes that must be resolved. A mixture of DNA fragments

[10] T. Maniatis and A. Efstratiadis, this volume, Article [38].
[11] P. G. N. Jeppesen, this volume, Article [39].
[12] G. G. Carmichael and G. K. McMaster, this volume, Article [47].
[13] G. S. Hayward, *Virology* 49, 342 (1972).
[14] R. A. Laskey, this volume, Article [45].
[15] K. J. Danna and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* 68, 2913 (1971).
[16] R. DeWachter and W. Fiers, Vol. 21, Part D, p. 167.
[17] U. Loening, *Biochem. J.* 102, 251 (1967).

ranging in size from 200 to 2000 nucleotide pairs can be resolved on a 13-cm long slab gel of 4% polyacrylamide by electrophoresis at 120 V for 3 hr. For adequate resolution of larger fragments, such as partial digestion products, the voltage or the time of electrophoresis or both should be increased. As an alternative, a gel with a larger pore size (i.e., lower percentage of acrylamide or agarose) can be employed.

Autoradiographic analysis of [^{32}P]DNA fragments can be achieved by wet-gel exposure of X-ray film, as described by DeWachter and Fiers,[16] or by dried-gel exposure. Wet-gel autoradiography is essential for purification of [^{32}P]DNA fragments from gels (see Section I,C). For wet-gel autoradiography, one of the glass plates enclosing the gel is removed; the gel, supported by the remaining glass plate, is covered with Saran wrap; a piece of medical X-ray film (e.g., Kodak Blue Brand or Kodak RP Royal X-omat) is laid atop the Saran wrap; and a clean glass plate is clamped on top of the film to ensure uniform contact. After an appropriate exposure time, the film is processed in Kodak D-19 developer (5 min) and Kodak Rapid-Fixer (5 min). As little as 2000 dpm of [^{32}P]DNA in an area of 1 mm^2 produces an easily visible spot on Kodak Blue Brand film after a 30-min exposure.

The alternate procedure of dried-gel autoradiography results in sharper bands because of a reduced scattering angle between the radioactive sample and the film. The gel can be dried on an automatic gel dryer[18] or by Maizel's modification[19] of a method described by Fairbanks *et al.*[20] The gel is first transferred to a sheet of Whatman 3MM paper, is placed gel-side up on a porous support (either a metal grid or a porous polyethylene sheet), and is covered with Saran wrap. With an automatic gel dryer, the assembly is placed gel-side up onto a prewarmed heating plate (about 80°), which has an integral vacuum manifold. The assembly is covered with a sheet of silicone rubber, which forms a seal about the gel when the vacuum system is activated. The combination of heat and vacuum dries a 14-cm × 13-cm × 1-mm gel in about 35 min. The dried gel is placed tightly against a piece of X-ray film for autoradiography.

C. Purification of DNA Fragments

Individual DNA fragments are conveniently purified by preparative gel electrophoresis, excision of gel segments containing DNA bands,[16] and recovery of the DNA from each segment. For purification of the [^{32}P]DNA

[18] For example, a Gel Slab Dryer, Model 224, manufactured by Bio-Rad Laboratories, Richmond, California.

[19] J. V. Maizel, Jr., *Methods Virol.* 5, 180.

[20] G. Fairbanks, Jr., C. Levinthal, and R. H. Reeder, *Biochem. Biophys. Res. Commun.* 20, 393 (1965).

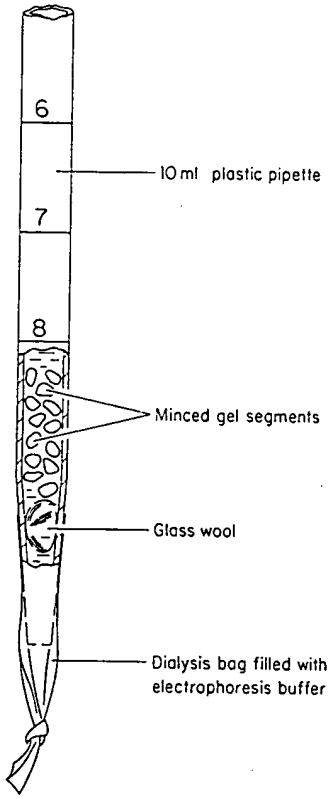

FIG. 1. Apparatus for recovery of DNA from gel segments by electrophoresis into a dialysis bag.

fragments in the model study, a wet-gel exposure of a preparative gel was made as described in Section I,B, except that labels written with ^{32}P-ink were placed at the corners of the slab before exposure.[16] The developed X-ray film was aligned on top of the gel by means of the radioactive labels, and the outline of the gel was traced onto the film. With the guidance of the tracing, the film was accurately aligned under the glass plate supporting the gel so that gel segments corresponding to DNA bands could be excised with a scalpel or razor blade.

For a description of general methods to recover DNA from gels, see Smith.[21] In the model study, recovery was accomplished by electrophoresis of the sample into a dialysis bag, a reliable method, which results in 80–90% recovery of DNA. A simple apparatus, illustrated in Fig. 1, consists of a short segment of a 10-ml plastic pipette with a glass

[21] H. O. Smith, this volume, Article [46].

wool plug in the tip. Attached to the pipette is a 3–4-cm long dialysis bag filled with electrophoresis buffer. The dialysis tubing should fit tightly over the tapered end of the pipette. After the pipette has been filled with electrophoresis buffer, minced gel segments containing a DNA fragment are transferred into the pipette and allowed to settle. The assembly is placed into a cylindrical gel apparatus with the dialysis bag toward the positive electrode so that the DNA will migrate into the bag during subsequent electrophoresis. The DNA recovered from the bag can be used directly or can be concentrated by precipitation in 0.03 *M* sodium acetate, pH 6.0, and 70% ethanol at −20°C. DNA purified from polyacrylamide gels in this way is suitable for further endonuclease digestion. In the model study, 90% of a partial digestion product about 1000 nucleotide pairs in length was recovered from a 1-ml volume of 4% polyacrylamide gel segments by electrophoresis at 150 V for 3 hr.

II. Ordering of Fragments by Partial Digestion

Analysis of partial digestion products to order fragments produced by cleavage of DNA with a restriction endonuclease employs the techniques described in Section I in the following steps.

1. The electrophoretic profile for products of complete digestion is established.
2. Individual partial digestion products from a large-scale digest are purified.
3. Each partial digestion product is redigested with an excess of enzyme and electrophoresed in parallel with a marker of completely digested DNA.
4. The resulting data are analyzed to construct a physical map.

The method is exemplified by the mapping of cleavage sites for *Hin*cII and *Hin*dIII on SV40 DNA, a circular molecule with a length of 5224[22] or 5226[23] nucleotide pairs. Figure 2 shows the major products of complete digestion of SV40 DNA with *Hin*cII (lane a) and with *Hin*dIII (lane b). Because they migrated off the gel, the two smallest fragments in the *Hin*cII digest, F and G, are not shown. The fragments are labeled alphabetically in order of decreasing size, and the length of each, derived from the nucleotide sequence of the DNA, is listed in Table I. Figure 2 (lane c) shows an example of an incomplete digest of SV40 DNA with

[22] W. Fiers, R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert, *Nature (London)* 273, 113 (1978).

[23] V. B. Reddy, B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman, *Science* 200, 494 (1978).

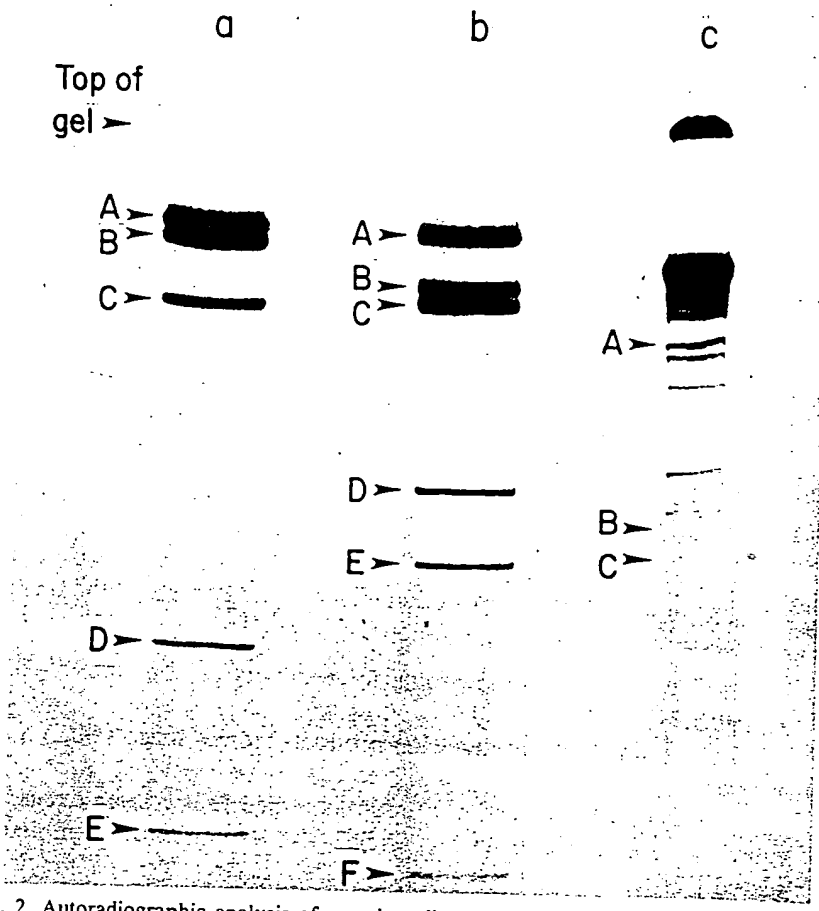

FIG. 2. Autoradiographic analysis of complete digests of SV40 DNA with *Hin*cII and *Hin*dIII and a partial digest with *Hin*dIII. (a) SV40 [^{32}P]DNA (0.1 μg) was digested with 0.25 unit of *Hin*cII in 10 μl of 10 m*M* Tris-HCl, pH 7.9, 7 m*M* $MgCl_2$, 60 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 0.5 mg/ml gelatin for 1 hr at 37°. A 5-μl aliquot was electrophoresed on a 13-cm long 4% polyacrylamide gel at 120 V for 2.5 hr and a dried-gel autoradiogram was prepared. (b) SV40 [^{32}P]DNA (0.1 μg) was digested with 0.25 unit of *Hin*dIII in 10 μl of 7 m*M* Tris-HCl, pH 7.4, 7 m*M* $MgCl_2$, 50 m*M* NaCl, 0.5 mg/ml gelatin for 1 hr at 37°. A 5-μl aliquot was analyzed as described for sample a. (c) SV40 [^{32}P]DNA (4 μg) was digested with 7.5 units of *Hin*dIII in a volume of 360 μl. At 10, 20, and 35 minutes 120 μl of the sample was removed and the reaction stopped by addition of SDS to a final concentration of 1% (w/v). A mixture of 1 μl from each sample was electrophoresed on a 4% polyacrylamide gel at 75 V for 20 hr adjacent to a *Hin*dIII complete digest marker and a dried-gel autoradiogram was prepared. Fragments are labeled alphabetically in order of decreasing size.

*Hin*dIII, the positions of the final products A, B, and C indicated by arrows. In theory, an incomplete digest of circular SV40 DNA with *Hin*dIII might contain up to thirty partial digestion products, including the

TABLE I
SIZES OF SV40 DNA FRAGMENTS PRODUCED BY CLEAVAGE WITH *Hin*dIII AND WITH *Hin*cII

*Hin*dIII product	Nucleotide pairs[a–c]	*Hin*cII product	Nucleotide pairs[a–c]
A	1768	A	1961,[b] 1963[c]
B	1169	B	1538
C	1099,[b] 1101[c]	C	1067
D	526	D	369
E	447	E	240
F	215	F	29
		G	20

[a] To account for the staggered breaks produced by *Hin*dIII, the number of nucleotide pairs in each fragment was taken to be one-half of the total nucleotides.
[b] Fiers *et al.*[22]
[c] Reddy *et al.*[23]

six unit-length linear species. Of these, seven are clearly resolved in the example, and one short fragment migrated off the gel.

Individual products of partial digestion with *Hin*cII and *Hin*dIII, purified by the method described in Section I,C, were redigested with the appropriate enzyme, and the products derived from each were identified by electrophoresis of the digest in parallel with a complete digest marker. In each case, the intact partial digestion product was also electrophoresed adjacent to the marker so that the distance of migration could be measured. Examples of electrophoretic analysis of partial products of *Hin*dIII digestion are shown in Fig. 3. When a partial product gives rise to a set of equimolar fragments, as judged from the intensities of the bands in the autoradiogram, analysis is usually straightforward. For example, the *Hin*dIII partial product in lane a of Fig. 3 yields fragments C and E when redigested (lane c), and the partial product in lane d yields E and F (lane f). One can conclude that C is contiguous to E and that E is contiguous to F in the SV40 genome. On the other hand, the fragment in lane g gives rise to A, B, C, and D, but clearly A and D are present in greater amount than B and C. Such a result is expected when the partial digestion product is actually a mixture of two different fragments that happened to comigrate in the preparative gel. Thus, in this example, one of the partial products is comprised of A and D, and the other of B and C. In other cases, redigestion may result in no apparent change in mobility of a putative partial digestion product because the fragment is actually a final product. This conclusion is confirmed if the putative partial product comigrates with a fragment in the complete digest marker.

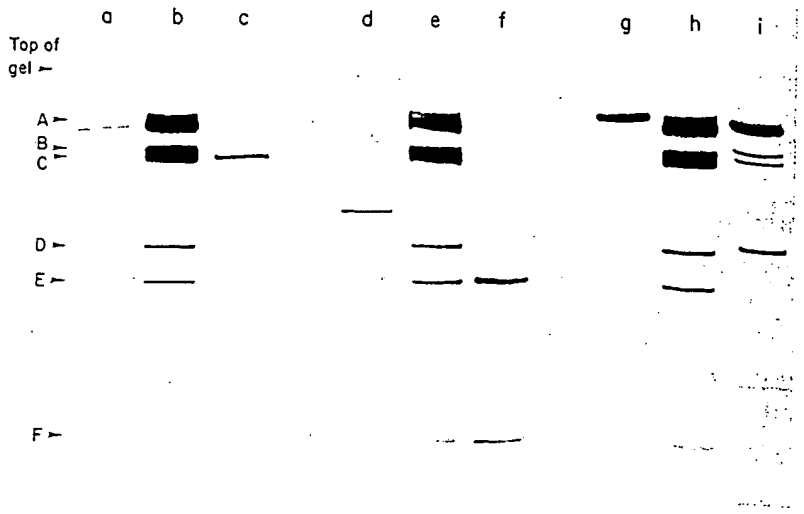

FIG. 3. Analysis of *Hin*dIII partial digestion products. Lanes b, e, and h are *Hin*dIII complete digest markers. Lane c is the result of redigestion of the partial digestion product in lane a; lane f is the digest of the partial product in lane d; and lane i is the digest of the partial product in lane g. Each partial digestion product was digested with *Hin*dIII by incubating 0.01 μg of DNA with 0.1 unit of enzyme in a volume of 30 μl for 1 hr at 37°. Samples were electrophoresed at 120 V for 2.5 hr on a 4% polyacrylamide gel and analyzed by dried-gel autoradiography.

Qualitative results based on comigration should be verified by comparing the size of each partial digestion product with the sum of sizes of the fragments derived from it. This is particularly important for identifying instances in which two partial digestion products that comigrate are also equimolar. In contrast to the example shown in Fig. 3 (lanes d and f), the two sets of final products derived from an equimolar mixture of partial products cannot be distinguished on the basis of intensities of the bands in the autoradiogram. However, the combined sizes of all the final products derived from such a mixture will be twice the estimated length of the putatively homogeneous partial product. Although a limited amount of information can be derived from analysis of an equimolar mixture of partial products, one can usually obtain sufficient data from less ambiguous cases to construct a physical map.

The length of a partial digestion product can be estimated on the basis of electrophoretic mobility,[1,24] using a plot of relative mobility versus log of fragment length. Figure 4 illustrates such a curve for unit-length linear SV40 DNA and the fragments in a *Hin*dIII digest of SV40 DNA, the

[24] A. J. Shatkin, J. D. Sipe, and P. Loh, *J. Virol.* 2, 989 (1968).

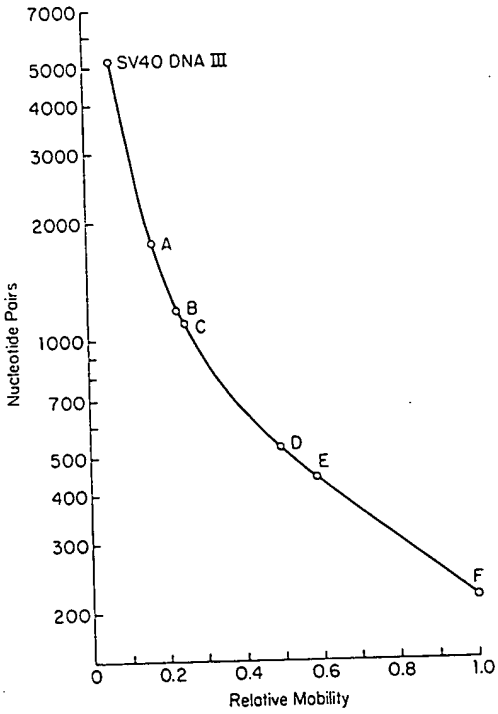

FIG. 4. Standard plot of relative mobility versus log of fragment length in a 4% polyacrylamide gel. Unit-length linear SV40 DNA and the fragments in a complete digest of SV40 DNA with *Hin*dIII were used as markers.

fragment lengths taken from the nucleotide sequence of the SV40 genome.[22,23] If DNA fragments of known length are not available as markers, the relative sizes of the fragments in a complete digest of [^{32}P]DNA can be determined from the relative radioactivities of the fragments assuming that radioactivity is directly proportional to length. The radioactivity in individual fragments can be measured by excising the bands from a preparative gel, as described in Section I,C. Each segment is dissolved in 0.2 ml of 30% hydrogen peroxide by incubation at 65° in a tightly capped scintillation vial and counted in a liquid scintillation spectrometer using a fluor for aqueous samples.[25]

Table II lists the results from analyses of several partial digestion products of both *Hin*dIII and *Hin*cII. Included are the estimated length of each partial digestion product, the final products derived from each, and the sum of lengths of the final products. For these data, the estimated length of each partial product agrees reasonably well with the sum of sizes

[25] For example, Aquasol, manufactured by New England Nuclear, Boston, Massachusetts.

TABLE II
ANALYSIS OF *Hin*dIII AND *Hin*cII PARTIAL DIGESTION PRODUCTS

*Hin*dIII product (relative mobility)[a]	Estimated size[b] (nucleotide pairs)	Final products	Sum of sizes[c] (nucleotide pairs)
0.39	670	E, F	662
0.18	1600	C, E	1546
0.14	2300	B, C	2268
		A, D	2294
0.13	2500	A, D, F	2509
0.12	2900	B, C, D	2794

*Hin*cII product (relative mobility)[a]	Estimated size[b] (nucleotide pairs)	Final products	Sum of sizes[c] (nucleotide pairs)
2.2	60	F, G	49
0.19	1500	C, D	1436
0.18	1600	B, G	1558
0.15	2000	A, F	1990
0.14	2300	B, D, E	2147
0.11	3200	A, C, F	3057

[a] Relative mobility was measured as distance migrated by fragment divided by distance migrated by *Hin*dIII F on the same gel.
[b] Estimated from electrophoretic mobility, using the plot in Fig. 4.
[c] The size of each final product was derived from the data of Fiers *et al.*[22]

of the final products. Each set of final products listed in Table II represents a group of fragments contiguous in the original DNA molecule. For example, based on the *Hin*dIII partial product of relative mobility 0.39, the final product E is contiguous to F in SV40 DNA. Analysis of the partial product of relative mobility 0.18 indicates that C and E are contiguous. Because these two groups of fragments share fragment E, they can be linked in the order C–E–F. The results in Table II can be arranged such that members common to each group of contiguous fragments are placed in overlapping positions, as shown in Fig. 5, to determine the order of all the fragments. The data thus lead to the construction of the two cleavage maps in Fig. 6, one for *Hin*dIII and the other for *Hin*cII.

III. Ordering of Fragments through Multiple Enzyme Digestion

One application of multiple enzyme digestion for ordering fragments[26] parallels the use of partial digests discussed in Section II. That is, if the products of enzyme α are to be ordered, purified products of several

[26] R. C. Yang, A. Van de Voorde, and W. Fiers, *Eur. J. Biochem.* 61, 119 (1976).

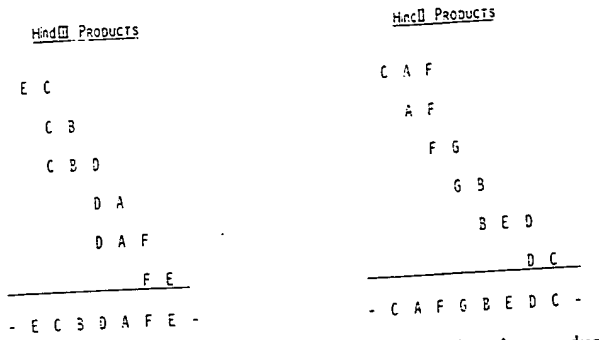

FIG. 5. Results of analysis of *Hin*dIII and *Hin*cII partial digestion products. Each group of contiguous fragments has been arranged so that members common to each group overlap to determine the complete order.

accessory enzymes (β, γ, δ, . . .) may be used in place of partial digestion products. For example, a fragment produced by enzyme β that includes three cleavage sites for enzyme α will yield, upon digestion with α, two α fragments and two fragments originating from the ends of the β fragment. If the end fragments can be distinguished from all α products, then one can conclude that the two observed α fragments are contiguous. Since the method requires that the β fragment include three or more α cleavage sites, accessory enzymes that produce large fragments are most useful. Moreover, several accessory enzymes are required to establish a sufficient number of sets of contiguous fragments to generate a map. The techniques required are similar to those utilized in Section II.

1. Electrophoretic profiles of fragments produced by several endonucleases (α, β, γ, δ, . . .) are determined.

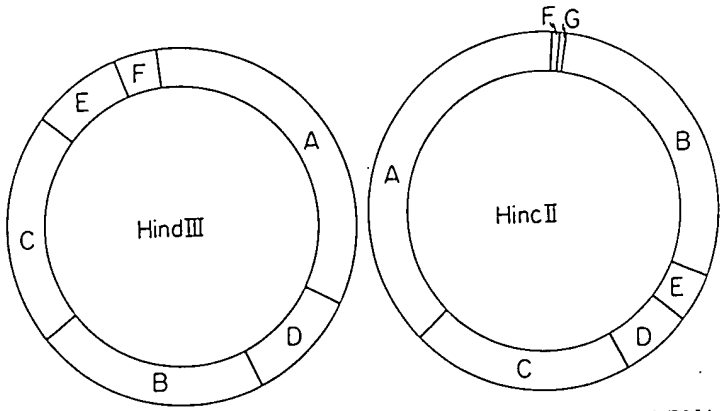

FIG. 6. Cleavage maps for the *Hin*dIII and *Hin*cII products of SV40 DNA.

2. Fragments produced by complete digestion with accessory enzymes β, γ, δ, . . . , are purified by the procedure described in Section I,C.

3. Each purified fragment is digested with enzyme α, and the limit products of α derived from each are identified by electrophoresis in parallel with a marker of α products.

4. Sets of contiguous fragments are arranged such that common members are placed in overlapping positions to construct a map.

This basic approach can also be used to correlate two existing cleavage maps. For example, the *Hin*cII and *Hin*dIII maps in Fig. 6 might be aligned by digesting purified *Hin*cII products with *Hin*dIII. The relative positions of the cleavage sites for the two enzymes can be deduced from the resulting data.

A second application of multiple enzyme digestion allows the correlation of independently constructed cleavage maps of two different enzymes. In contrast to the first approach, this method relies on electrophoretic analysis of double digests and usually requires no purification of fragments. A particularly simple analysis results if each accessory enzyme used in this method recognizes only a single cleavage site in the DNA.

The first step of the procedure involves characterizing the cleavage products of each accessory enzyme. In the model study, the endonucleases *Taq*I and *Bam*HI are used to relate the *Hin*dIII and *Hin*cII cleavage maps that were established in Section II (Fig. 6). *Taq*I and *Bam*H1 each cleaves SV40 DNA at a single site, as shown in Fig. 7. The single bands in lanes a (*Taq*I digest) and b (*Bam*H1 digest) correspond to unit-length linear SV40 DNA. The distance between the cleavage sites for the two enzymes can be estimated as described in Section II by sequential digestion of ^{32}P-labeled SV40 DNA and quantitation of the radioactivity in the products, A and B (Fig. 7, lane c). Fragment A accounts for 58% of the total radioactivity and fragment B for 42%. Since SV40 DNA contains about 5200 nucleotide pairs, fragment A is approximately 3000 nucleotide pairs long and fragment B is about 2200 nucleotide pairs long.

The next step involves double digestion with each accessory enzyme and the enzymes *Hin*cII and *Hin*dIII. If optimal reaction conditions for two enzymes are similar, the enzymes can be used simultaneously. For example, for double digestion of SV40 DNA with *Bam*H1 and *Hin*dIII, 0.02 μg of SV40[^{32}P]DNA was incubated with 0.1 unit of *Hin*dIII and 0.1 unit of *Bam*H1 at 37°C for 1 hr in 20 μl of 7 m*M* Tris-HCl, pH 7.9, 7 m*M* $MgCl_2$, 50 m*M* NaCl, 7 m*M* 2-mercaptoethanol, and 0.5 mg/ml gelatin. Likewise, *Hin*cII and *Bam*H1 can be used together. On the other hand, since conditions optimal for *Taq*I require incubation at 50° with no NaCl, sequential digestion is necessary.

Top of gel

FIG. 7. *Taq*I, μg) was digeste m*M* 2-mercapto a 1.3% agarose [^{32}P]DNA (0.02 6 m*M* $MgCl_2$, 5 Analysis was th as described for sample was inc

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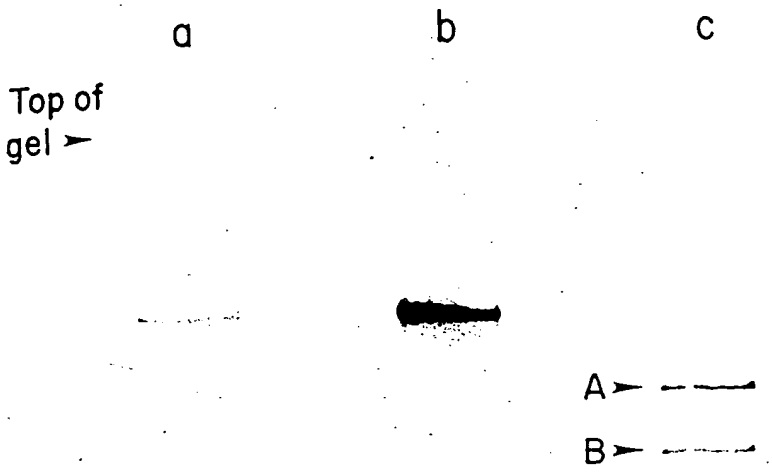

FIG. 7. *Taq*I, *Bam*H1, and *Taq*I/*Bam*H1 digests of SV40 DNA. (a) SV40 [^{32}P]DNA (0.02 μg) was digested with 0.1 unit of *Taq*I in 20 μl of 10 m*M* Tris-HCl, pH 8.0, 6 m*M* $MgCl_2$, 6 m*M* 2-mercaptoethanol, 0.5 mg/ml gelatin at 50° for 1 hr. The sample was electrophoresed on a 1.3% agarose gel for 3 hr at 40 V and analyzed by dried-gel autoradiography. (b) SV40 [^{32}P]DNA (0.02 μg) was digested with 0.1 unit of *Bam*H1 in 20 μl of 6 m*M* Tris-HCl, pH 7.9, 6 m*M* $MgCl_2$, 50 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 0.5 mg/ml gelatin at 37° for 1 hr. Analysis was the same as for sample a. (c) SV40 [^{32}P]DNA (0.02 μg) was digested with *Taq*I as described for sample a. Then 1 μl of 1 *M* NaCl and 0.1 unit of *Bam*H1 were added and the sample was incubated at 37° for 1 hr. Analysis was the same as for sample a.

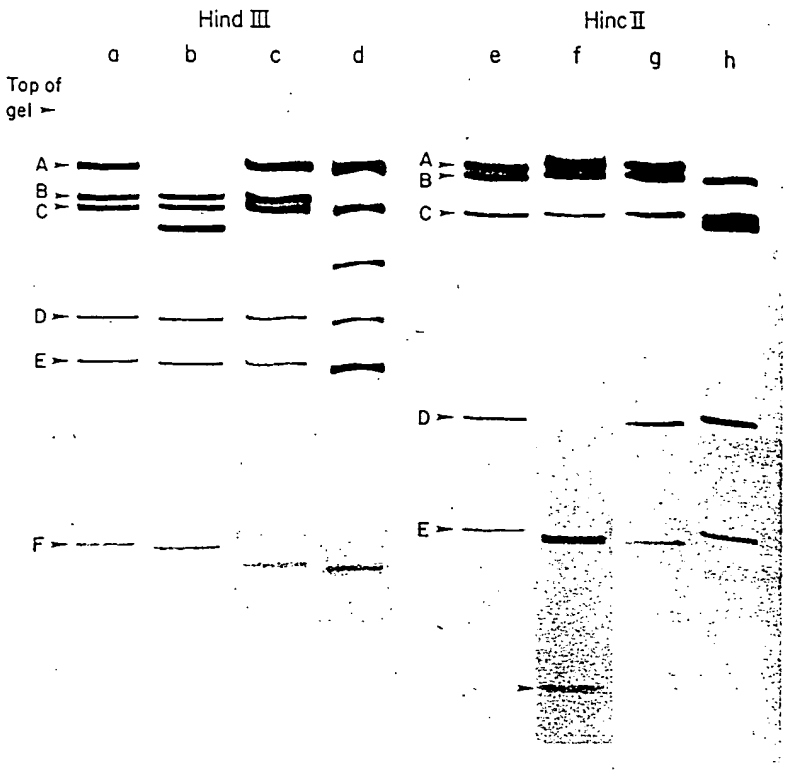

FIG. 8. Double digests of *Hin*dIII and *Hin*cII products with *Bam*HI and *Taq*I. Lanes a and c are complete *Hin*dIII digest markers; lanes e and g are complete *Hin*cII digest markers. Lane b, 0.02 μg of SV40 [^{32}P]DNA was incubated with 0.1 unit of *Hin*dIII and 0.1 unit of *Bam*HI at 37° for 1 hr in 20 μl of 7 m*M* Tris-HCl, pH 7.9, 7 m*M* $MgCl_2$, 50 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 0.5 mg/ml gelatin. Lane d, 0.02 μg of SV40 [^{32}P]DNA was digested with *Taq*I as for sample a in Fig. 7. One microliter of 1 *M* NaCl and 0.1 unit of *Hin*dIII were added and incubation was continued for 1 hr at 37°. Lane f, 0.02 μg of SV40 [^{32}P]DNA was digested with 0.1 unit of *Bam*HI and 0.1 unit of *Hin*cII in 20 μl of 6 m*M* Tris-HCl, pH 7.9, 7 m*M* $MgCl_2$, 50 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 0.5 mg/ml gelatin at 37° for 1 hr. Lane h, 0.02 μg of SV40 [^{32}P]DNA was digested with *Taq*I as described in the legend to Fig. 7. One microliter of 1 *M* NaCl and 0.1 unit of *Hin*cII were added and incubation was continued at 37° for 1 hr. All samples were electrophoresed on 4% polyacrylamide gels at 120 V for 2.5 hr and analyzed by dried-gel autoradiography.

Fragments resulting from double digestion are analyzed electrophoretically to localize the cleavage sites for *Taq*I and *Bam*HI within specific *Hin*dIII and *Hin*cII fragments. In Fig. 8, a comparison between a complete *Hin*dIII digest (lane a) and a *Bam*HI/*Hin*dIII double digest (lane b) indicates that in the double digest *Hin*dIII A is missing, and a new band below

TABLE III
PRODUCTS OF MULTIPLE ENZYME DIGESTION

Fragment cleaved by *Bam*H1	Size[a] (nucleotide pairs)	Estimated sizes[b] of products (nucleotide pairs)
*Hin*dIIIA	1768	900 900
*Hin*cIID	369	240 130
*Taq*I linear DNA	5224	3000 2200
Fragment cleaved by *Taq*I	**Size[a] (nucleotide pairs)**	**Estimated sizes[b] of products (nucleotide pairs)**
*Hin*dIIIB	1169	740 430
*Hin*cIIA	1961	1000 1000

[a] Derived from the data of Fiers *et al.*[22]
[b] Estimated from electrophoretic mobility, using the plot in Fig. 4.

*Hin*dIII C is present. Similarly, for the *Taq*I/*Hin*dIII digest (lane d), *Hin*dIII B is missing and a new band below *Hin*dIII C appears. Therefore, the cleavage site for *Bam*H1 is within the *Hin*dIII A fragment and that for *Taq*I is within the *Hin*dIII B fragment. The other two double digests (lanes f and h) indicate that *Bam*H1 cleaves within the *Hin*cII D fragment and that *Taq*I cleaves within the *Hin*cII A fragment. Since both the *Hin*dIII A and the *Hin*cII D fragments contain the *Bam*H1 recognition site, they must overlap; likewise, the *Hin*dIII B and the *Hin*cII A fragments overlap. These results roughly determine the relative orientations of the two maps.

In order to relate the two maps precisely, the exact locations of the *Bam*H1 and *Taq*I sites on the *Hin*dIII and *Hin*cII maps must be determined. Each double digest contains two new products, the sizes of which can be estimated on the basis of electrophoretic mobility, using the plot in Fig. 4. The simplest result of double digestion, not exemplified in this model study, is that the two new products migrate as distinct bands. A second possibility is shown in Fig. 8, lane b. The new product between the *Hin*dIII C and D fragments has an estimated length of 900 nucleotide pairs, about half the length of the parent fragment, *Hin*dIII A (1768 nucleotide pairs). The fact that the new band is more dense than the band corresponding to the longer *Hin*dIII C fragment indicates that the band is actually a doublet. *Bam*H1 therefore cleaves the *Hin*dIII A fragment near its center to produce two comigrating fragments. The same conclusion can be reached for the *Taq*I/*Hin*cII digest (lane h), in which *Taq*I cleaves the *Hin*cII A fragment (1961 nucleotide pairs) to yield two fragments, each

TABLE IV
CLEAVAGE OF FRAGMENTS A AND B FROM A *TaqI/Bam*H1 DIGEST WITH *Hin*cII AND *Hin*dIII

*TaqI/Bam*H1 fragment	Products of digestion with *Hin*cII		Products of digestion with *Hin*dIII	
	Identifiable *Hin*cII products	Estimated sizes[a] of additional products	Identifiable *Hin*dIII products	Estimated sizes[a] of additional products
A	B, E, F, G	240 1000	C, E, F	430 900
B	C	130 1000	D	740 900

[a] Estimated from electrophoretic mobility, using the plot in Fig. 4.

about 1000 nucleotide pairs in length. In contrast to these cases, the *TaqI/Hin*dIII and *Bam*H1/*Hin*cII digests exemplify another possible result, namely, that only one new band appears but, adjudged from the intensity of the band in the autoradiogram, it cannot be a doublet. Shown in lane d of Fig. 8, the *Hin*dIII B fragment (1169 nucleotide pairs) is cleaved by *Taq*I to produce a new fragment about 740 nucleotide pairs long that migrates between C and D. Since the predicted length of the companion new fragment is about 430 nucleotide pairs, it should migrate near *Hin*dIII E (450 nucleotide pairs long). Consistent with this prediction is the observation that the *Hin*dIII E band is broader and denser than the D fragment in the double digest, indicating that it is indeed a doublet. Likewise, *Bam*HI cleaves the *Hin*cII D fragment (lane f) to yield the new fast-migrating fragment (about 140 nucleotide pairs), indicated by an arrow and a second fragment that comigrates with the *Hin*cII E fragment (about 240 nucleotide pairs). These results are summarized in Table III.

The data are used to construct a cleavage map by comparing the known distance between the *Taq*I and *Bam*H1 cleavage sites with the possible distances calculated from the lengths of the double digestion products. For example, since *Taq*I produces two fragments from *Hin*dIII B, 740 and 430 nucleotide pairs in length, the cleavage site might be nearer the B–D junction or nearer the B–C junction (see Fig. 6). As summarized in Table III, *Bam*H1 cleaves the *Hin*dIII A fragment near its center. The shorter distance between the *Bam*H1 and *Taq*I cleavage sites, about 2200 nucleotide pairs, should equal the sum of half of *Hin*dIII A (900 nucleotide pairs), *Hin*dIII D (526 nucleotide pairs) and either the 740- or 430-nucleotide pair fragment derived from *Hin*dIII B. The former possibility yields a total of 2166 nucleotide pairs whereas the latter yields only 1856. The *Taq*I site is, therefore, near the B–C junction. These arguments locate

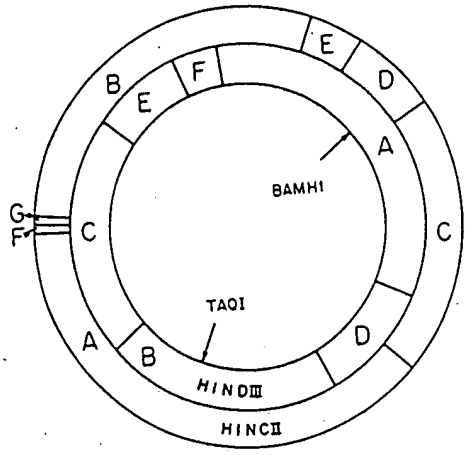

FIG. 9. Composite physical map of SV40 DNA, including the cleavage sites for *Taq*I, *Bam*H1, *Hin*dIII, and *Hin*cII.

the *Bam*H1 and *Taq*I cleavage sites on the *Hin*dIII map. With regard to the *Hin*cII map, *Bam*H1 cleaves the D fragment asymmetrically to yield fragments 130 and 240 nucleotide pairs long and *Taq*I cleaves the A fragment near its center to yield two fragments about 1000 nucleotide pairs in length. The two possible arrangements of the *Taq*I and *Bam*H1 cleavage sites in the *Hin*cII map yield a distance of either 2307 (if *Bam*H1 cleaves near the D–E junction) or 2197 (if *Bam*H1 cleaves near the D–C junction), so that *Bam*H1 probably cleaves *Hin*cII D near the D–C junction. These results can be verified by purifying fragments A and B from a *Taq*I/*Bam*H1 double digest and cleaving each with *Hin*cII and with *Hin*dIII. As shown in Table IV, the digestion of fragment B with *Hin*cII yields *Hin*cII C, a new product of 1000 nucleotide pairs, and a new product of 130 nucleotide pairs. Digestion of fragment B with *Hin*dIII yields *Hin*dIII D, a new product of 900 nucleotide pairs, and a third product 740 nucleotide pairs long. Thus, the conclusions drawn from the original double digests are sound.

The double digest data generate the composite physical map of the SV40 genome shown in Fig. 9. Not only are the *Taq*I and *Bam*H1 sites located within specific *Hin*dIII and *Hin*cII fragments, but also the relationship between the *Hin*dIII and *Hin*cII cleavage sites is established.